

Genes of purine biosynthesis from *Ashbya gossypii* and the use thereof in microbial riboflavin synthesis

- 5 The present invention relates to genes of purine biosynthesis from *Ashbya gossypii* and to the use thereof in riboflavin synthesis.
- 10 Vitamin B2, also called riboflavin, is essential for humans and animals. Vitamin B2 deficiency is associated with inflammations of the mucous membranes of the mouth and throat, itching and inflammations in the skin folds and similar cutaneous lesions, conjunctival inflammations, reduced visual accuracy and clouding
- 15 of the cornea. Babies and children may experience cessation of growth and loss of weight. Vitamin B2 therefore has economic importance, especially as vitamin supplement in cases of vitamin deficiency and as supplement to animal feed. It is also employed for coloring foodstuffs, for example in mayonnaise, icecream,
- 20 blancmange etc.
- Vitamin B2 is prepared either chemically or microbially (see, for example, Kurth et al. (1996) riboflavin, in: Ullmann's Encyclopedia of industrial chemistry, VCH Weinheim). In the
- 25 chemical preparation process, riboflavin is, as a rule, obtained as pure final product in multistage processes, it being necessary to employ relatively costly starting materials such as, for example, D-ribose. An alternative to the chemical synthesis of riboflavin is the preparation of this substance by
- 30 microorganisms. The starting materials used in this case are renewable raw materials such as sugars or vegetable oils. The preparation of riboflavin by fermentation of fungi such as *Eremothecium ashbyii* or *Ashbya gossypii* is known (The Merck Index, Windholz et al., eds. Merck & Co., page 1183, 1983), but
- 35 yeasts such as, for example, *Candida*, *Pichia* and *Saccharomyces*, or bacteria such as, for example, *Bacillus*, clostridia or corynebacteria, have also been described as riboflavin producers.
- EP 405370 describes riboflavin-overproducing bacterial strains
- 40 obtained by transformation of the riboflavin biosynthesis genes from *Bacillus subtilis*. These genes described therein, and other genes involved in vitamin B2 biosynthesis from prokaryotes are unsuitable for a recombinant riboflavin preparation process using eukaryotes such as, for example, *Saccharomyces cerevisiae* or
- 45 *Ashbya gossypii*.

DE 44 20 785 describes six riboflavin biosynthesis genes from *Ashbya gossypii*, and microorganisms transformed with these genes, and the use of such microorganisms for riboflavin synthesis.

5 It is possible with these processes to generate producer strains for microbial riboflavin synthesis. However, these producer strains often have metabolic limitations which cannot be eliminated by the inserted biosynthesis genes or are sometimes induced thereby. Such producer strains are sometimes unable to  
10 provide sufficient substrate for saturating some steps in the biosynthesis, so that the biosynthetic capacity of some segments of metabolism cannot be fully exploited.

15 It is therefore desirable to enhance further sections of metabolic pathways, thereby to eliminate metabolic bottlenecks and thus further optimize the microorganism employed for the microbial riboflavin synthesis (producer strains) in respect of their ability for riboflavin synthesis. It is desirable to  
20 identify the enhancing sections of the complex metabolism and to enhance these in a suitable way.

The present invention relates to novel proteins of purine biosynthesis, the genes therefor and the use thereof for  
25 microbial riboflavin synthesis.

Purine metabolism (for a review, see, for example, Voet, D. and Voet, J.G., 1994, *Biochemie*, VCH Weinheim, pages 743-771; Zalkin, H. and Dixon, J.E., 1992, *De novo purine nucleotide*  
30 *biosynthesis*, in: *Progress in nucleic acid research and molecular biology*, Vol. 42, pages 259-287, Academic Press) is a part of the metabolism which is essential for all life forms. Faulty purine metabolism may in humans lead to serious diseases (e.g. gout). Purine metabolism is moreover an important target for treating  
35 oncoses and viral infections. Numerous publications have appeared describing substances which intervene in purine metabolism for these indications (as review, for example Christopherson, R.I. and Lyons, S.D., 1990, *Potent inhibitors of de novo pyrimidine and purine biosynthesis as chemotherapeutic agents*, *Med. Res.*  
40 *Reviews* 10, pages 505-548).

Investigations on the enzymes involved in purine metabolism (Smith, J.L., *Enzymes in nucleotide synthesis*, 1995, *Curr. Opin. Struct. Biol.* 5, 752-757) aim to develop novel  
45 immunosuppressives, antiparasitic or antiproliferative medicines (*Biochem. Soc. Transact.* 23, pages 877-902, 1995).

These medicines are normally not naturally occurring purines, pyrimidines or compounds derived therefrom.

5 The present invention relates to a protein having the polypeptide sequence depicted in SEQ ID NO:2 or a polypeptide sequence obtainable from SEQ ID NO:2 by substitution, insertion or deletion of up to 15% of the amino acids, and having the enzymatic activity of a phosphoribosyl-pyrophosphate synthetase.

10 The sequence depicted in SEQ ID NO:2 is the gene product of the KPR1 gene (SEQ ID NO:1) obtained from *Ashbya gossypii*.

15 The invention further relates to a protein having the polypeptide sequence depicted in SEQ ID NO:5 or a polypeptide sequence obtainable from SEQ ID NO:5 by substitution, insertion or deletion of up to 10% of the amino acids, and having the enzymatic activity of a glutamine-phosphoribosyl-pyrophosphate amidotransferase.

20 The sequence depicted in SEQ ID NO:5 is the gene product of the ADE4 gene (SEQ ID NO:3) obtained from *Ashbya gossypii*.

25 The invention further relates to a protein having the polypeptide sequence depicted in SEQ ID NO:8 or a polypeptide sequence obtainable from SEQ ID NO:8 by substitution, insertion or deletion of up to 20% of the amino acids, and having the enzymatic activity of an IMP dehydrogenase.

30 The sequence depicted in SEQ ID NO:8 and 9 is the gene product of the GUA1 gene (SEQ ID NO:7) obtained from *Ashbya gossypii*.

35 The invention further relates to a protein having the polypeptide sequence depicted in SEQ ID NO:11 or a polypeptide sequence obtainable from SEQ ID NO:11 by substitution, insertion or deletion of up to 10% of the amino acids, and having the enzymatic activity of a GMP synthetase.

40 The sequence depicted in SEQ ID NO:11 is the gene product of the GUA2 gene (SEQ ID NO:10) obtained from *Ashbya gossypii*.

45 The invention further relates to a protein having the polypeptide sequence depicted in SEQ ID NO:13 or a polypeptide sequence obtainable from SEQ ID NO:13 by substitution, insertion or

deletion of up to 10% of the amino acids, and having the enzymatic activity of a phosphoribosyl-pyrophosphate synthetase.

The sequence depicted in SEQ ID NO:13 is the gene product of the  
5 KPR2 gene (SEQ ID NO:12) obtained from *Ashbya gossypii*.

These gene products mentioned can be modified by conventional methods of gene technology, such as site-directed mutagenesis, so that particular amino acids are replaced, additionally inserted  
10 or deleted. Amino acid residues are normally (but not exclusively) replaced by those of similar volume, charge or hydrophilicity/ hydrophobicity in order not to lose the enzymatic properties of the gene products. In particular, modifications of the amino acid sequence in the active center frequently results  
15 in a drastic alteration in the enzymatic activities. However, modifications of the amino acid sequence and other, less essential sites are often tolerated.

It is possible with the novel proteins  
20

1. for up to 15, preferably up to 10 and particularly preferably up to 5, % of the amino acids to be modified, by comparison with sequences depicted in the sequence listing, in the case  
25 of the gene product of the AgKPR1 gene;
2. for up to 10 and particularly preferably up to 5% of the amino acids to be modified, by comparison with the sequences depicted in the sequence listing, in the case of the gene  
30 product of the AgADE4 gene;
3. for up to 20, preferably up to 15, particularly preferably up to 10 and especially preferably up to 5, % of the amino acids to be modified, by comparison with the sequences depicted in  
35 the sequence listing, in the case of the gene product of the AgGUA1 gene;
4. for up to 10 and particularly preferably up to 5% of the amino acids to be modified, by comparison with the sequences depicted in the sequence listing, in the case of the gene  
40 product of the AgGUA2 gene;
5. for up to 10%, preferably up to 7% and particularly preferably up to 5%, of the amino acids to be modified, by comparison with the sequences depicted in the sequence  
45 listing, in the case of the gene product of the AgKPR2 gene.

10076157 021502

Preferred proteins are those which, while they still have the relevant enzymatic activity, have altered regulation. Many of these enzymes are subject to a strong control of the activity by intermediates and final products (feedback inhibition). This leads to the activity of the enzymes being restricted as soon as sufficient final product is present.

However, in the case of producer strains, this economic control in the physiological state often results in it being impossible to increase the productivity beyond a certain limit. Elimination of such feedback inhibition results in the enzymes retaining their activity, irrespective of the final product concentration, and thus metabolic bottlenecks are bypassed. This in the end leads to a marked increase in riboflavin biosynthesis.

Preferred novel proteins are those no longer inhibited by secondary products of metabolic pathways (derived from products of the enzymes). Particularly preferred novel proteins are those no longer inhibited by intermediates of purine biosynthesis, in particular by purine bases, purine nucleosides, purine nucleotide 5'-monophosphates or purine nucleotide 5'-diphosphates or purine nucleotide 5'-triphosphates. Particularly preferred novel proteins are those with subsequent modifications of the amino acid sequence and all combinations of amino acid sequence modifications which comprise these subsequent modifications.

Modifications of the amino acid sequence of the AgKPR1 gene product:

Lysine at position 7 replaced by valine  
 Aspartate at position 52 replaced by histidine  
 Leucine at position 131 replaced by isoleucine  
 Aspartate at position 186 replaced by histidine  
 Alanine at position 193 replaced by valine  
 Histidine at position 196 replaced by glutamine

Modifications of the amino acid sequence of the AgADE4 gene product:

Aspartate at position 310 replaced by valine  
 Lysine at position 333 replaced by alanine  
 Alanine at position 417 replaced by tryptophan

10076157.021502

The following Examples describe the preparation of the novel proteins and nucleic acids and the use thereof for producing microorganisms with increased riboflavin synthesis.

5 Example 1:

Production of a genomic gene bank from *Ashbya gossypii* ATCC10895

Genomic DNA from *Ashbya gossypii* ATCC10895 can be prepared by  
 10 conventional methods as described, for example, in WO9703208. The  
 genomic gene bank can be constructed starting from this DNA by  
 conventional methods (e.g. Sambrook, J. et al. (1989) Molecular  
 cloning: a laboratory manual, Cold Spring Harbor Laboratory Press  
 or Ausubel, F.M. et al. (1994) Current protocols in molecular  
 15 biology, John Wiley and sons) in any suitable plasmids or  
 cosmids, such as, for example, SuperCos1 (Stratagene, La Jolla,  
 USA).

Example 2:

20 Cloning of the gene for PRPP synthetase from *Ashbya gossypii*  
 ATCC10895 (AgKPR1)

Cloning of the gene for PRPP synthetase from *Ashbya gossypii*  
 25 (AgKPR1) can take place in two steps. In the first step, it is  
 possible with the following oligonucleotides to amplify a defined  
 region of the KPR1 gene from genomic DNA from *Ashbya gossypii* by  
 PCR:

30 KPR5: 5'- GATGCTAGAGACCGCGGGTGCAAC -3'  
 KPR3: 5'- TGTCCGCCATGTCGTCTACAATAATA -3'

The PCR can be carried out by a conventional method. The  
 resulting 330 bp DNA fragment can be cloned by conventional  
 35 methods into the vector pGEMT (Promega, Madison, USA) and be  
 sequenced.

A genomic cosmid gene bank can be screened by conventional  
 40 methods using this nucleotide sequence as probe. A 1911 bp  
 PstI-HindIII fragment of a cosmid which gives a signal with this  
 probe can then be subcloned into the vector pBluescript SK+  
 (Stratagene, La Jolla, USA). The KPR1 gene and incomplete ORFs  
 which show homology with the UBC6 and UBP9 genes of *Saccharomyces*  
 45 *cerevisiae* are located on this fragment.

The PRPP synthetase KPR2 and the putative PRPP synthetase KPR4 from *Saccharomyces cerevisiae* are the enzymes which are most closely related, with similarities of 80.2% and 79.6% respectively, to the PRPP synthetase from *Ashbya gossypii*. The KPR2 and KPR4 genes from *Saccharomyces cerevisiae* have 67.6% and 67.8%, respectively, similarity with the KPR1 gene from *Ashbya gossypii*. Other enzymes and genes from other organisms are distinctly more different from the KPR1 gene and from the PRPP synthetase from *Ashbya gossypii*.

10

The sequence comparisons can be carried out, for example, with the Clustal algorithm with the aid of the PAM250 weighting table or the Wilbur-Lipman DNA alignment algorithm (as implemented, for example, in the MegAlign 3.06 program package supplied by DNASTar). It is not possible with the oligonucleotide pair described to amplify the genes for the different PRPP synthetases from *Saccharomyces cerevisiae*.

It is also possible to use the probe to find a further clone from the gene bank. This second clone showed a gene which likewise codes for a PRPP synthetase. This gene is called AgKPR2 and is distinctly different from AgKPR1. AgKPR2 shows 66% identity with AgKPR1 at the amino acid level. The AgKPR2 gene (SEQ ID NO:12) was compared with all proteins of the Swissprot database. The maximum similarity shown by this protein (88% identity and 95% similarity) is with the KPR3 gene product from *Saccharomyces cerevisiae*. The gene product of the AgKPR1 gene is responsible for the predominant part of the PRPP synthetase activity in *Ashbya gossypii*. Disruption of the AgKPR1 gene of *Ashbya gossypii* (analogous to the disruption of other *Ashbya* genes as in the descriptions in Examples 6-8) results in a distinctly reduced enzyme activity: in place of 22 U/mg of protein now only 3 U/mg of protein. See Example 13 for the analysis. Examples 11, 13 and 15 relate to the AgKPR1 gene, but studies of these types can also be carried out with AgKPR2.

#### Example 3:

Cloning of the gene for glutamine-PRPP amidotransferase from *Ashbya gossypii* ATCC10895 (AgADE4)

The cloning of the gene for glutamine-PRPP amidotransferase from *Ashbya gossypii* (AgADE4) can take place in two steps.

In the first step, it is possible with the following oligonucleotides to amplify a defined region of the AgADE4 gene from genomic DNA of *Ashbya gossypii* by PCR:

ADE4A: 5'- ATATCTTGATGAAGACGTTACCGT -3'

ADE4B: 5'- GATAATGACGGCTTGGCCGGAAGA -3'

- 5 The PCR can be carried out by a conventional method. The resulting 360 bp DNA fragment can be cloned by conventional methods into the vector pGEMT (Promega, Madison, USA) and then be sequenced.
- 10 This sequence can be used as probe to screen a genomic cosmid gene bank by conventional methods. It is then possible to subclone a 5369 bp HindIII fragment from a cosmid which gives a signal with this probe into the vector pBluescript SK+ (Stratagene, La Jolla, USA). The AgADE4 gene and the gene for the
- 15 Ashbya homolog for the mitochondrial ABC transporter ATM1 from *Saccharomyces cerevisiae* and another open reading frame whose function is unknown are located on this fragment.

- The AgADE4 gene product (glutamine-PRPP amidotransferase) shows
- 20 the most evident similarity with the ADE4 gene products from *Saccharomyces cerevisiae* and *Saccharomyces kluyveri* (81% and 86.3% respectively). The corresponding genes show only 68.8% and 72%, respectively, homology, however. The similarity with other glutamine-PRPP amidotransferases is distinctly less (e.g. only
- 25 27.5% similarity with the corresponding enzyme from *Bacillus subtilis*). The sequence comparisons can be carried out as described in Example 2.

- It is not possible with the described pair of oligonucleotides to
- 30 amplify the ADE4 genes from *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*.

#### Example 4:

- 35 Cloning of the gene for inosine-monophosphate dehydrogenase from *Ashbya gossypii* ATCC10895 (AgGUA1)

Cloning of the gene for inosine-monophosphate dehydrogenase from *Ashbya gossypii* (AgGUA1) can take place in two steps.

40

In the first step, it is possible with the following oligonucleotides to amplify a defined region of the AgGUA1 gene from genomic DNA from *Ashbya gossypii* by PCR:

45

IMP5: 5'- GGCATCAACCTCGAGGAGGCGAACC -3'



IMP3: 5'- CAGACCGGCCTCGACCAGCATCGCC - 3'

The PCR can be carried out by a conventional method. The resulting 230 bp DNA fragment can be cloned by conventional methods into the vector pGEMT (Promega, Madison, USA) and then be sequenced.

This sequence can be used as probe to screen a genomic cosmid gene bank by conventional methods. A 3616 bp ApaI fragment from a cosmid which gives a signal with this probe can be subcloned into the vector pBluescript SK+ (Stratagene, La Jolla, USA). The coding region of the AgGUA1 gene is 1569 bp long and is interrupted by a 161 bp-long intron. The intron boundaries (5' splice site AGGTATGT and 3' splice site CAG) can be verified by cloning and sequencing of AgGUA1cDNA.

AgGUA1 is the first gene described from *Ashbya gossypii* having an intron.

The AgGUA1 gene product (IMP dehydrogenase) shows the most evident similarity with the 4 IMP dehydrogenases from *Saccharomyces cerevisiae* (similarities between 67% and 77.2%). The similarity with other IMP dehydrogenases is distinctly less. The sequence comparisons can be carried out as described in Example 2. *Ashbya gossypii* appears to have only one gene for this enzyme. This can be shown by Southern blotting with genomic DNA from *Ashbya gossypii* using the abovementioned probe.

The gene from *Saccharomyces cerevisiae* which codes for the IMP dehydrogenase (IMH3) which has most similarity with the AgGUA1 gene product has a similarity of 70.2% with the AgGUA1 gene. It is not possible with the described pair of oligonucleotides to amplify this gene from *Saccharomyces cerevisiae*.

Example 5:

Cloning of the gene for guanosine-monophosphate synthetase from *Ashbya gossypii* ATCC10895 (AgGUA2)

Cloning of the gene for guanosine-monophosphate synthetase from *Ashbya gossypii* (AgGUA2) can take place in two steps.

In the first step, it is possible with the following oligonucleotides to amplify a defined region of the AgGUA2 gene from genomic DNA from *Ashbya gossypii* by PCR:

GUA2A: 5'- TGGACCGGGCGGTGTTTCGAGTTGGG -3'

GUA2B: 5'- AGGCTGGATCCTGGCTGCCTCGCGC -3'

The PCR can be carried out by a conventional method. The resulting 750 bp DNA fragment can be cloned by conventional methods into the vector pBluescript SK+ (Stratagene, La Jolla, USA) and then be sequenced.

This sequence can be used as probe to screen a genomic cosmid gene bank by conventional methods. A 2697 bp ClaI-EcoRV fragment from a cosmid which gives a signal with this probe can then be subcloned into the vector pBluescript SK+ (Stratagene, La Jolla, USA).

The AgGUA2 gene product (GMP synthetase) shows the most evident similarity with GMP synthetase from *Saccharomyces cerevisiae* (similarity 86.6%). The genes for the GMP synthetases from *Saccharomyces cerevisiae* and *Ashbya gossypii* show 71.2% homology. The similarity of the AgGUA2 gene product with other GMP synthetases is distinctly less. The sequence comparisons can be carried out as described in Example 2.

It is not possible with the described pair of oligonucleotides to amplify the GMP synthetase gene from *Saccharomyces cerevisiae*.

Example 6:

Disruption of the AgADE4 gene from *Ashbya gossypii* ATCC10895

Disruption of a gene means destroying the functionality of a genomic copy of the gene either by (a) deleting part of the gene sequence, or by (b) interrupting the gene by inserting a piece of foreign DNA into the gene or by (c) replacing part of the gene by foreign DNA. Any foreign DNA can be used, but it is preferably a gene which brings about resistance to any suitable chemical. Any suitable resistance genes can be used for disruption of genes.

A gene which confers resistance to G418 can be used to disrupt the AgADE4 gene from *Ashbya gossypii* ATCC10895. It is possible for this to be the kanamycin resistance gene from TN903 under the control of the TEF promoter of *Ashbya gossypii* (see, for example, Yeast 10, pages 1793-1808, 1994, W09200379). The gene is flanked 5' and 3' by several cleavage sites for restriction endonucleases, thus constructing a cassette which allows any desired constructions of gene disruptions by conventional methods of in vitro manipulation of DNA.

The internal HincII fragment of AgADE4 (between positions 2366 and 2924) can be replaced by a resistance cassette as outlined above. The resulting construct is called ade4::G418.

- 5 The resulting plasmid can be replicated in E.coli. The BamHI / BglIII fragment of the construct ade4::G418 can be prepared, purified by agarose gel electrophoresis and subsequent elution of the DNA from the gel (see Proc. Natl. Acad. Sci. USA 76, 615-619, 1979) and employed for transforming *Ashbya gossypii*.

10

Ashbya gossypii can be transformed by protoplast transformation (Gene 109, 99-105, 1991), but preferably by electroporation (BioRad Gene Pulser, conditions: cuvettes with slit widths 0.4 mm, 1500V, 25µF, 100Ω). Transformed cells are selected from

15

G418-containing solid medium.

Resulting G418-resistant clones can be examined by conventional methods of PCR and Southern blot analysis to find whether the

20

genomic copy of the AgADE4 gene is in fact destroyed. Clones whose AgADE4 gene is destroyed are purine-auxotrophic.

Example 7:

25

Disruption of the AgGUA1 gene from *Ashbya gossypii* ATCC10895

See Example 6 for a description of the principle of disruption of genes, the use of a resistance cassette and the transformation of *Ashbya gossypii*.

30

The internal XhoI / KpnI fragment of AgGUA1 (between positions 1620 and 2061) can be replaced by a resistance cassette as outlined above. The resulting construct is called gual::G418.

35

The resulting plasmid can be replicated in E.coli. The XbaI / BamHI fragment of the construct gual::G418 can be prepared, purified by agarose gel electrophoresis and subsequent elution of the DNA from the gel and employed for transforming *Ashbya gossypii*.

40

Resulting G418-resistant clones can be examined by conventional methods of PCR and Southern blot analysis to find whether the genomic copy of the AgGUA1 gene is in fact destroyed. Clones whose AgGUA1 gene is destroyed are guanine-auxotrophic.

45

10076157.024502

## Example 8:

Disruption of the AgGUA2 gene from *Ashbya gossypii* ATCC10895

- 5 See Example 6 for a description of the principle of disruption of genes, the use of a resistance cassette and the transformation of *Ashbya gossypii*.

- 10 The internal SalI fragment of AgGUA2 (between positions 1153 and 1219) can be replaced by a resistance cassette as outlined above. The resulting construct is called *gua2::G418*.

- 15 The resulting plasmid can be replicated in *E.coli*. The XbaI / BamHI fragment of the construct *gua2::G418* can be prepared, purified by agarose gel electrophoresis and subsequent elution of the DNA from the gel and employed for transforming *Ashbya gossypii*.

- 20 Resulting G418-resistant clones can be examined by conventional methods of PCR and Southern blot analysis to find whether the genomic copy of the AgGUA2 gene is in fact destroyed. Clones whose AgGUA2 gene is destroyed are guanine-auxotrophic.

25 Example 9:

Cloning of the GAP promoter from *Ashbya gossypii*

- 30 The gene for glyceraldehyde-3-phosphate dehydrogenase from *Ashbya gossypii* (AgGAP) can be cloned by generally customary screening of a genomic *Ashbya gossypii* cosmid gene bank (see Example 1, with a probe which was constructed from information on the sequence of the GAP gene from *Saccharomyces cerevisiae*).

- 35 The 5' nontranslated region of the gene (-373 to -8 region relative to the translation start) was assumed to be promoter. 2 cleavage sites for the restriction endonuclease NotI were inserted flanking this sequence. In this region there are the bona fide TATA Box (nt 224-230), two sequence sections (nt 43-51 and 77-85) which correspond to the GCR1 binding element, and a  
40 sequence section (nt 9-20) whose complement partially corresponds to the RAP1 binding element of *Saccharomyces cerevisiae* (see, for example, Johnston, M. and Carlson, M. (1992) pp.193-281 in *The molecular biology and cellular biology of the yeast Saccharomyces: Gene expression*, Cold Spring Harbor Laboratory  
45 Press). The promoter cassette constructed in this way can be placed as easily portable expression signal in front of any desired gene for overexpression in *Ashbya gossypii* and results in

pronounced overexpression of genes in *Ashbya gossypii*, as shown in Example 11.

Example 10:

- 5 Construction of plasmids having genes under the control of the GAP promoter from *Ashbya gossypii*

10 In order to introduce the GAP promoter cassette 5' of the coding region of the AgADE4 gene, a unique NotI cleavage site (recognition sequence GCGGCCGC) was inserted by conventional methods (e.g. Glover, D.M. and Hames, B.D. (1995) DNA cloning Vol.1, IRL press) 8 bp 5' of the ATG start codon.

15 The GAP promoter cassette can then be inserted via NotI into this position. An analogous procedure can be used for cloning the GAP promoter cassette 5' of the coding region of the genes AgKPR1, AgGUA1, AgGUA2 and for variants of the genes AgADE4, AgKPR1, AgGUA1 and AgGUA2.

20

Expression of the genes which harbor the GAP promoter cassette 5' of the coding region in *Ashbya gossypii* is controlled by the GAP promoter.

25 Example 11:

Overexpression of genes in *Ashbya gossypii* under the control of the GAP promoter

- 30 Transformation of *Ashbya gossypii* with the DNA constructs described in Example 10 can be carried out as described in Example 6. The recipient clones can preferably, but not exclusively, be those which, before the transformation to be carried out here, harbor a disruption of the gene to be
- 35 overexpressed. Thus, for example, the *Ashbya gossypii* mutant which is described in Example 6 and harbors an *ade4::G418* mutation can be transformed with a GAP-ADE4 construct described in Example 10. Integration of the construct into the genome can be verified by Southern blot analysis. The resulting clones no
- 40 longer have a G418 resistance gene (and are thus G418-sensitive) and are purine-prototrophic. Overexpression can be demonstrated by Northern blot analysis or detection of the enzymatic activity (as described in Example 12). On expression of the AgADE4 gene under the natural promoter, 0.007 U/mg of protein can be
- 45 detected. On expression of the AgADE4 gene under the GAP promoter, 0.382 U/mg of protein can be detected.

A sequence section of the coding region of the AgADE4 gene can be used as probe. An analogous procedure can be used with AgKPR1, AgGUA1, AgGUA2 and for variants of all these genes. In addition, combinations of one of these genes together with other genes can  
5 be introduced in this way into the genome of *Ashbya gossypii*.

The wild type *Ashbya gossypii* has a specific PRPP synthetase activity of 22 U/mg of protein (see Example 13 for analysis of the PRPP synthetase). On expression of the AgKPR1 gene with the  
10 GAP promoter, 855 U/mg of protein is detectable.

#### Example 12:

Variants of the AgADE4 gene product (glutamine-PRPP  
15 amidotransferase) no longer subject to feedback inhibition by purines or intermediates of purine synthesis.

Glutamine-PRPP amidotransferases are subject to feedback inhibition by purine nucleotides. This inhibition is found in  
20 numerous organisms (see, for example, Switzer, R.L. (1989) Regulation of bacterial Glutamine Phosphoribosylpyrophosphate Amidotransferase, in: Allosteric enzymes pp. 129-151, CRC press, Boca Raton).

25 The glutamine-PRPP amidotransferase from *Ashbya gossypii* is likewise inhibited by AMP or GMP (see Figure). The activity of glutamine-phosphoribosyl-pyrophosphate amidotransferase from *Ashbya gossypii* can be measured as described in Messenger and Zalkin (1979) J. Biol. Chem. 254, pages 3382-3392.

30 Modified glutamine-phosphoribosyl-pyrophosphate amidotransferases no longer inhibited by purines can be constructed. It is evident that overexpression of such deregulated enzymes will enhance purine metabolism distinctly more than overexpression of enzymes  
35 subject to feedback inhibition. Alterations in the sequence of the AgADE4 gene can be brought about by conventional methods (e.g. Glover, D.M. and Hames, B.D. (1995) DNA cloning Vol.1, IRL press). It is possible, for example, for the following amino acids in glutamine-phosphoribosyl-pyrophosphate amidotransferase  
40 to be replaced:

The codon which codes for aspartate at position 310 can be replaced by a codon which codes for valine. The codon which codes  
45 for lysine at position 333 can be replaced by a codon which codes for alanine. The codon which codes for alanine at position 417 can be replaced by a codon which codes for tryptophan. It is

additionally possible to construct AgADE4 genes which harbor combinations of these substitutions.

- 5 All enzymes which carry D310V, K333A, A417W or any combination of substitutions which comprise D310V or K333A show diminished feedback inhibition by AMP and GMP (see Figure). This can be shown, for example, by expressing the enzymes in *Ashbya gossypii* (see Example 11).

10 Example 13:

Variants of the AgKPR1 gene product (PRPP synthetase) no longer subject to feedback inhibition by purines or intermediates of purine synthesis.

- 15 PRPP synthetases are subject to feedback inhibition by purines, pyrimidines and amino acids. This inhibition is found in numerous organisms (see, for example, Gibson, K.J. et al. (1982) *J. Biol. Chem.* 257, 2391-2396; Tatibana, M. et al. (1995) *Adv., Enzyme Regul.* 35, 229-249 and papers quoted therein).

- In clinical medical research there are descriptions of cases of hereditary gout based on enhanced purine biosynthesis. The molecular cause thereof is what is called superactivity of human  
25 PRPP synthetase (see, for example, *Amer. J. Med.* 55 (1973) 232-242; *J. Clin. Invest.* 96 (1995) 2133-2141; *J. Biol.* 268 (1993) 26476-26481). The basis thereof may be a mutation which leads to the enzyme no longer being subject to feedback inhibition by purines.

- 30 The activity of the PRPP synthetase from *Ashbya gossypii* can be measured as described in *Anal. Biochem.* 98 (1979) 254-263 or *J. Bacteriol.* 174 (1992) 6852-6856. The specific activity (U/mg) is defined via the amount of resulting product (nmol/min/g of protein).

- 35 It is possible to construct modified PRPP synthetases no longer inhibited by purines. It is evident that overexpression of such deregulated enzymes enhances purine metabolism distinctly more than does overexpression of enzymes subject to feedback inhibition. Modifications of the sequence of the AgKPR1 gene may  
40 be brought about by conventional methods (e.g. Glover, D.M. and Hames, B.D. (1995) *DNA cloning Vol. 1*, IRL press). It is possible, for example, to exchange the following amino acids of the PRPP synthetase:

- The codon which codes for leucine at position 131 can be replaced  
45 by a codon which codes for isoleucine. The codon which codes for histidine at position 196 can be replaced by a codon which codes for glutamine.

All enzymes which have one of these amino acid exchanges (L131I or H196Q) show a reduced feedback inhibition by purines. Figure 2 shows this by the example of ADP.

This can be shown after expression of the corresponding enzymes 5 in *Ashbya gossypii*. This can be carried out in accordance with Example 11.

Example 14:

- 10 Variants of the AgGUA1 gene product (IMP dehydrogenase) no longer subject to feedback inhibition by purines or intermediates of purine synthesis.

Example 15:

- 15 Effects of the enhancement and/or optimization of enzymes of purine metabolism and their genes on riboflavin production in *Ashbya gossypii*

- 20 The original strain *Ashbya gossypii* ATCC10895 can be tested for riboflavin productivity in shaken flasks, comparing with clones which are derived therefrom and harbor chromosomal copies of genes under the control of the GAP promoter (as described in Example 11). It is possible to use for this purpose 300 ml shaken flasks with 20 ml of YPD medium (Sambrook, J. et al. (1989)
- 25 Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press), incubating at a temperature of 28°C.

- After 2 days, the control strain produces on average 14.5 mg of riboflavin per l of culture broth. Strains which overexpress 30 genes for enzymes of purine metabolism (as shown, for example, in Example 11), or overexpress genes for optimized enzymes of purine metabolism (for example as in Examples 12, 13 and 14), produce more riboflavin. Thus, the strain which overexpresses
- 35 AgADE4D310VK333A (Example 12) produces on average 45.4 mg of riboflavin per l of culture broth in 2 days.

- The strain which overexpresses AgKPR1 with the GAP promoter produces not 14 mg/l (like the WT) but 36 mg/l riboflavin. The 40 strain which overexpresses AgKPR1H196Q with the GAP promoter produces 51 mg/l riboflavin.

Figure 1:

- Measurement of the activity of Gln-PRPP amidotransferase from *A. 45 gossypii* and of modified forms of the enzyme as a function of the concentration of adenosine 5'-monophosphate (AMP) and guanosine 5'-monophosphate (GMP).



WT: Gln-PRPP amidotransferase

A417W: Gln-PRPP amidotransferase, alanine at position 417 replaced by tryptophan.

5 K333A: Gln-PRPP amidotransferase, lysine at position 333 replaced by alanine.

D310VK333A: Gln-PRPP amidotransferase, aspartate at position 310 replaced by valine and lysine at position 333 replaced by alanine.

10

Figure 2:

Measurement of the activity of the PRPP synthetase from *A. gossypii* and of modified forms of the enzyme as a function of the concentration of adenosine 5'-diphosphate (ADP)

15 WT: PRPP synthetase

L131I: PRPP synthetase, leucine at position 131 replaced by isoleucine

H196Q: PRPP synthetase, histidine at position 196 replaced by glutamine

20 H196Q, L131I: PRPP synthetase, histidine at position 196 replaced by glutamine and leucine at position 131 replaced by isoleucine

25

30

35

40

45

20250720 15:45:00